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Protection of Hamsters by Venezuelan Equine Encephalitis Virus Candidate Vaccine V3526 against Lethal Challenge by Mosquito Bite and Intraperitoneal Injection

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Abstract. In an attempt to improve the current live, attenuated vaccine (TC-83) for Venezuelan equine encephalitis virus (VEEV), specific mutations associated with attenuation of VEEV in rodent models were inserted into a full-length cDNA clone of the Trinidad donkey strain of VEEV by site-directed mutagenesis. Because some viruses have been reported to be more pathogenic when introduced by mosquito bite than the same virus introduced by needle inoculation, there were concerns that the presence of mosquito saliva, or changes in the virus caused by replication in a mosquito, might allow the virus to overcome the protective effects of prior vaccination with V3526. Therefore, we determined if hamsters vaccinated with V3526 were protected from challenge with the virulent Trinidad donkey strain of VEEV. All non-vaccinated hamsters died after intraperitoneal challenge or after being fed on by VEEV-inoculated *Aedes taeniorhynchus*. In contrast, hamsters vaccinated with V3526 were resistant to intraperitoneal challenge and infection by VEEV-infected *Ae. taeniorhynchus*. Therefore, the V3526 candidate vaccine elicits protection against VEEV infection by mosquito bite.

INTRODUCTION

In efforts to develop an improved live-attenuated vaccine for Venezuelan equine encephalitis virus (VEEV), specific mutations associated with attenuation of VEEV in rodent models were identified and inserted into a full-length cDNA clone of VEEV to produce selected isogenic strains containing one or more attenuating mutations.[1–3] These mutations were evaluated for their potential as a live, attenuated VEEV vaccine. One of these, the V3526 strain, which contains a deletion of the furin cleavage site in PE2 and a suppressor mutation in E1,[4] protects mice, hamsters, and nonhuman primates challenged either by intraperitoneal inoculation or by aerosol.[5–7] In addition, this strain replicates less efficiently in potential mosquito vectors and does not revert to virulence after multiple passages in mosquitoes.[7]

In nature, most infections with VEEV are caused by the bite of an infective mosquito. Studies have indicated that some viruses, when introduced by mosquitoes or along with mosquito saliva into a vertebrate host, may be more pathogenic than virus introduced alone. These studies include increased viremia in chipmunks infected with La Crosse virus by mosquito bite compared with those infected by needle inoculation[8] and mice inoculated with Cache Valley virus in the same location as mosquitoes had just fed compared with mice inoculated with virus alone.[9] In addition, Schneider and others[10] showed that mortality rates were higher in mice inoculated with West Nile virus in the same location as mosquitoes that had just fed compared with mice inoculated with virus alone. Therefore, vaccination with the V3526 vaccine candidate might not protect against virulent VEEV if virus was introduced by the bite of an infectious mosquito. To evaluate the potential for mosquito-introduced virus to overcome the immunity induced by vaccination with V3526, we vaccinated hamsters and challenged them either intrperitoneally or subcutaneously, or by allowing VEEV-inoculated mosquitoes to feed on them.

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MATERIALS AND METHODS

Mosquitoes. The Medical and Veterinary Entomology Research Laboratory (MAVERL) laboratory strain of *Aedes taeniorhynchus* was used in these studies. This strain has been in a colony for more than 40 years and was derived from mosquitoes collected in the late 1950s in Florida. Mosquitoes were held at 26°C with a 16:8 hour light:dark photoperiod and reared as described by Gargan and others.[11]

Aedes taeniorhynchus is considered a natural vector of VEEV in the Americas,[12] and this strain is highly competent for the epizootic IAB strain of VEEV (Turell MJ, unpublished data).[13] Two- to 6-day-old female *Ae. taeniorhynchus* were inoculated intrathoracically[14] with 0.3 μL of a suspension containing approximately $10^{4.3}$ plaque-forming units (PFU)/mL ($10^{0.8}$ PFU/mosquito) of the virulent Trinidad donkey strain of VEEV and then placed in a 0.9-liter cardboard container with netting over the open end. The inoculated mosquitoes were held in an incubator maintained at 26°C with a 16:8 hour light:dark photoperiod and provided apple slices as a carbohydrate source. After 11 days of extrinsic incubation, 30 of the VEEV-inoculated mosquitoes were placed individually in 0.9-liter cardboard containers and allowed to feed on the hamsters from groups 1 and 2 (one mosquito/hamster) as described below.

Virus and virus assay. Plaque titers for specimens were determined on Vero cell monolayers grown in six-well plastic cell culture plates. Serial 10-fold dilutions of each specimen were added to wells (0.1 mL/well). After a 1-hour absorption period, a nutrient overlay (Eagle's basal medium with Earle's salts, 7% heat-inactivated fetal bovine serum, 0.75% agarose, and antibiotics) was added to each well and the plates were incubated at 35°C for 2 days. Cells were then stained with 1 mL of the above medium except that 5% of commercial neutral red (Invitrogen, Carlsbad, CA) (final concentration = 160 μg/mL) was used in place of the fetal bovine serum and antibiotics. Plaques were counted the next day.

Experimental design. After 2 weeks of acclimation in the laboratory, female hamsters (90–100 g) were divided into two groups. The 25 hamsters in group 1 were inoculated intraperitoneally with 0.2 mL of diluent (10% heat-inactivated fetal bovine serum in medium 199 with Earle's salts, $NaHCO_3$, and antibiotics), and the 25 hamsters in group 2

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14. ABSTRACT

In an attempt to improve upon the current live, attenuated vaccine (TC-83) for Venezuelan equine encephalitis virus (VEEV), the V3526 vaccine candidate strain of VEEV was prepared by site-directed mutagenesis. Because studies indicate that virus introduced by mosquito bite may be more pathogenic than the same virus introduced by needle inoculation, there were concerns that the presence of mosquito saliva, or changes in the virus due to replication in a mosquito, might allow the virus to overcome the protective effects of prior vaccination with V3526. Therefore, we determined if hamsters vaccinated with V3526 were protected from challenge with virulent Trinidad donkey strain of VEEV. All non-vaccinated hamsters succumbed after intraperitoneal challenge or after being fed on by VEEV-inoculated Ochlerotatus taeniorhynchus. In contrast, hamsters vaccinated with V3526 were resistant to intraperitoneal challenge and infection by VEEV-infected Oc. taeniorhynchus. Therefore, the V3526 candidate vaccine elicits protection against VEEV infection by mosquito bite. In efforts to develop an improved live-attenuated vaccine for Venezuelan equine encephalitis virus (VEEV), specific mutations associated with attenuation of VEEV in rodent models were identified and inserted into a full-length cDNA clone of VEEV to produce selected isogenic strains containing one or more attenuating mutations.These were evaluated for their potential as a live, attenuated VEEV vaccine, and the V3526 strain, containing a deletion of the furin cleavage site in PE2 as well as a suppressor mutation in E1, was shown to protect mice, hamsters, and nonhuman primates challenged either by intraperitoneal (I.P.) inoculation or by aerosol. In addition, this strain replicates less efficiently in potential mosquito vectors and does not revert to virulence after multiple passages in mosquitoes. In nature, most infection with VEEV would be due to the bite of an infective mosquito. Several studies have indicated that virus introduced into a vertebrate host along with mosquito saliva may be more pathogenic than virus introduced alone. These include increased viremia in chipmunks infected with La Crosse virus by mosquito bite as compared to those infected by needle inoculationand mice inoculated with Cache Valley virus in the same location as mosquitoes had just fed as compared to mice inoculated with virus alone.nt VEEV if virus was introduced by the bite of an infectious mosquito. To evaluate the potential for mosquito-introduced virus to overcome the immunity induced by vaccination with V3526, we vaccinated hamsters and challenged them either I.P. or by allowing VEEV-inoculated mosquitoes to feed on them.

15. SUBJECT TERMS

Venezuelan equine encephalitis fever, VEE, V3526 vaccine, efficacy, laboratory animals, hamsters

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were inoculated intraperitoneally with 0.2 mL of a 1:50 dilution of Lot # 1114.08 of the candidate VEEV vaccine, V3526, with a final titer of $10^{3.4}$ PFU/mL ($10^{2.7}$ PFU inoculated/hamster) in the same diluent used to inoculate group 1. Hamsters were held for 45 days and then challenged either by intraperitoneally inoculation with 0.2 mL ($10^{3.6}$ PFU/hamster) of the virulent Trinidad donkey strain (10 hamsters from each group) or by allowing a single *Ae. taeniorhynchus* that had been inoculated 11 days previously with the Trinidad donkey strain of VEEV to feed on each hamster (15 hamsters from each group). The dose of virus used for the challenge, $10^{3.6}$ PFU/hamster, is nearly identical to the dose of virus injected by a VEEV-inoculated *Ae. taeniorhynchus*, $10^{3.7}$ PFU.[15] For mosquito feedings, hamsters were anesthetized with a ketamine-acepromazine-xylazine suspension. The anesthetized hamsters were placed on top of a 0.5-liter cardboard cage with netting over the top, which contained one VEEV-inoculated mosquito. Each of the mosquitoes was triturated in 1 mL of diluent immediately after the 30-minute feeding attempt to confirm the presence of blood and then frozen at –70°C until tested by plaque assay to confirm the presence of virus. Hamsters were observed for 21 days.

In a second experiment designed to examine more subtle effects of vaccination or infection, 20 additional female hamsters (90–100 g) were allowed to acclimatize for 1 week and then an IPTT-200 remote temperature chip (BioMedic Data Systems, Inc. Seaford, DE) was inserted subcutaneously into each hamster. After acclimatizing for an additional 2 weeks, we remotely detected the temperature of each hamster with a Pocket Scanner model No. DAS-5007 (BioMedic Data Systems, Inc.), weighed the hamster, and then inoculated it with vaccine or diluent (10 hamsters each) as described above. We measured temperature and weight for four consecutive days and then weekly for 4 weeks. The weights and temperatures of individual hamsters differed and produced a relatively large standard deviation. To reduce this internal variation, we calculated the net gain or loss in weight and temperature for each hamster in that group individually for each day after vaccination. These deviations from the starting weights and temperatures were then used to calculate the standard deviations of the daily mean temperatures and weights. At 32 days after vaccination, each hamster was bled from the superior vena cava (0.5 mL), and held for an additional 3 days. Sera obtained from these blood samples were tested by a plaque-reduction neutralization test[16] to determine their ability to neutralize the Trinidad donkey strain of VEEV. At this time, three hamsters from each group were inoculated subcutaneously with 0.2 mL ($10^{3.5}$ PFU/hamster) of the virulent Trinidad donkey strain, three hamsters from each group were anesthetized and each hamster was fed upon by one *Ae. taeniorhynchus* that had been inoculated with the Trinidad donkey strain of VEEV 7 days previously, and three hamsters from each group were anesthetized and each hamster was fed upon by a single uninfected *Ae. taeniorhynchus* to serve as a room control. Each of the hamsters was weighed and its temperature was taken before being bled (0.1 mL from the superior vena cava) and then daily for 5 days.

TABLE 1

Protection of hamsters against lethal challenge of the Trinidad donkey strain of Venezuelan equine encephalitis virus (VEEV)*

Vaccine	Challenge	No.	% Survival	Days to death (SD)
V3526	IP†	10	100	NA
V3526	Mosquito‡	15	100	NA
Diluent	IP†	10	0	4.8 (0.4)
Diluent	Mosquito‡	15	0	4.8 (0.8)

* IP = intraperitoneal; NA = not applicable.
† Hamsters were inoculated IP with 0.2 mL of suspension containing $10^{3.6}$ plaque-forming units (PFU) of VEEV ($10^{4.3}$ PFU/mL).
‡ Hamsters were fed upon by one mosquito that had been inoculated with VEEV 11 days earlier.

This research was conducted in compliance with the Animal Welfare Act and other federal statues and regulations relating to animals and experiments involving animals and adheres to principles stated in the *Guide for the Care and Use of Laboratory Animals*, National Research Council, 1996. The facility where this research was conducted is fully accredited by the Association for the Assessment and Accreditation of Laboratory Animal Care International.

RESULTS

Protection study. All 10 mock-vaccinated hamsters (group 1) died (or were humanely killed when moribund) within 5 days after intraperitoneally inoculation of $10^{3.6}$ PFU of the Trinidad donkey strain of VEEV (Table 1). In contrast, none of 10 V3526-vaccinated hamsters (group 2) died or became ill after intraperitoneal inoculation with the same challenge virus. Similarly, all 15 of the group 1 hamsters died (or were humanely killed when moribund) within 5 days after being fed upon by one VEEV-inoculated *Ae. taeniorhynchus*, and none of 15 V3526-vaccinated hamsters (group 2) died or became ill after being fed upon by one VEEV-inoculated *Ae. taeniorhynchus*. Mean time to death for both groups of mock-vaccinated hamsters (needle-inoculated or mosquito-inoculated) was 4.8 days, with standard deviations of 0.4 and 0.8 days, respectively, for the two groups. Testing of the in-

TABLE 2

Effect of vaccination with V3526 on temperature and weight in hamsters

	Days after vaccination								
Vaccine	0*	1	2	3	4	11	18	25	32
					Temperature (°C)†				
V3526	36.9 (0.3)	36.9 (0.2)	37.4 (0.2)	36.6 (0.2)	36.7 (0.2)	36.8 (0.2)	36.5 (0.2)	36.9 (0.1)	36.3 (0.1)
Diluent	36.9 (0.2)	37.0 (0.3)	37.3 (0.1)	36.4 (0.2)	36.8 (0.2)	37.1 (0.2)	36.9 (0.1)	37.1 (0.2)	36.4 (0.1)
					Weight (g)‡				
V3526	115.1	113.7 (0.4)	114.6 (0.7)	114.7 (0.5)	115.1 (0.5)	119.9 (1.3)	123.0 (2.0)	126.2 (1.8)	127.1 (2.0)
Diluent	115.1	114.4 (0.8)	116.4 (0.9)	116.6 (0.9)	116.9 (0.6)	118.7 (0.8)	119.5 (1.0)	120.6 (1.3)	123.0 (1.3)

* Measured approximately 15 minutes before vaccination.
† Mean temperature (SD) by day after vaccination measured remotely from an implanted chip.
‡ Mean body weight (SD) by day after vaccination.

TABLE 3
Protection of hamsters against lethal challenge with the Trinidad donkey strain of Venezuelan equine encephalitis virus (VEEV)*

Vaccine	Challenge	No.	% Survival	Viremia by day after challenge				Days to death (SD)
				1	2	3	4	
V3526	SC†	3	100	0	0	0	0	NA
V3526	Mosquito‡	3§	100	0	0	0	0	NA
Diluent	SC†	3	0	5.7 (0.2)¶	7.0 (0.7)	6.6 (0.5)	NA	4.0 (0.0)
Diluent	Mosquito‡	3	0	4.6 (1.4)	5.6 (0.8)	5.4 (1.2)	6.3 (0.1)	4.7 (0.6)

* SC = subcutaneously; NA not applicable because all hamsters were dead on day 4.
† Hamsters were inoculated SC with 0.1 mL of suspension containing $10^{3.5}$ plaque-forming units (PFU) of VEEV ($10^{4.5}$ PFU/mL).
‡ Hamsters were fed upon by one mosquito that had been inoculated with VEEV 7 days earlier.
§ Four hamsters originally fed on by mosquitoes, but one died during bleeding and was not counted as a challenge death.
¶ Mean (SD) of the $\log_{10}$ PFU/mL of blood by day after challenge. Level of detection was 10^2 PFU/mL.

dividual mosquitoes that had fed on these hamsters indicated that all mosquitoes were infected and that the mean titer and standard error was $10^{6.4\pm0.1}$ PFU/mosquito for both groups of mosquitoes.

In the second experiment, designed to look at more subtle effects of vaccination or infection, we did not observe any effect of vaccination with the V3526 vaccine candidate on either temperature or weight (Table 2). All hamsters vaccinated with the V3526 vaccine had neutralizing antibodies, with titers $\geq$ 1:80 when bled 32 days after vaccination, and none of the hamsters vaccinated with diluent contained detectable antibodies. After challenge with virulent VEEV by either subcutaneous inoculation or bite by an infected mosquito, survival results were similar to those observed in the first experiment (all non-vaccinated hamsters were dead or humanely killed by day 5). However, during this experiment, one of the vaccinated hamsters died during a blood draw from the superior vena cava. Because this hamster had normal temperature and did not have a detectable viremia at any time, its death was attributed to hemorrhage from the bleed rather than to infection. Similarly, virus was not detected in the blood from any of the other vaccinated hamsters. In contrast, each of the challenged, non-vaccinated hamsters developed a viremia $\geq 10^{4.7}$ PFU/mL. The weights and temperatures of the challenged vaccinated hamsters were similar to those of the nonchallenged controls (Figures 1 and 2). In contrast, each of the challenged, nonvaccinated hamsters developed a febrile response (Figure 1) and lost weight (mean = 17 g/hamster) (Figure 2). No difference was observed between the vaccinated hamsters challenged by subcutaneous needle inoculation or mosquito bite. However, temperature elevation, weight loss, and viremia were slightly greater in nonvaccinated hamsters infected subcutaneously than in those infected by mosquito bite (Figures 1 and 2, Table 3).

DISCUSSION

Vaccination with the V3526 candidate live-attenuated VEEV vaccine protected hamsters against lethal challenge with virulent VEEV by either intraperitoneal or subcutaneous inoculation or by mosquito bite. Although this vaccine candidate was shown previously to protect mice, hamsters, and nonhuman primates, there was concern that virus introduced by an infected mosquito might be more pathogenic than virus administered by needle inoculation.

Several studies indicate that mosquito-transmitted virus may be more pathogenic than virus introduced by needle inoculation,[8–10,17] including either increased viremias[8,9] or in-

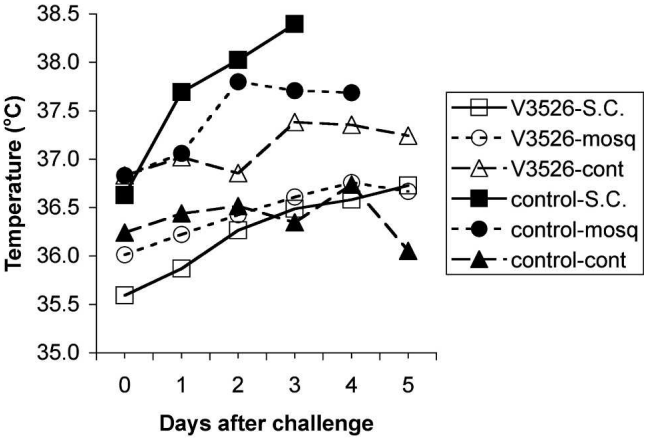

FIGURE 1. Temperature in hamsters by day after challenge with virulent Venezuelan equine encephalitis virus (VEEV). Hamsters were vaccinated with either live, attenuated V3526 vaccine (V3526) or diluent (control) 35 days before being challenged subcutaneously with $10^{3.5}$ plaque-forming units of the Trinidad donkey strain of VEEV (S.C.) fed upon by a mosquito that had been inoculated with the Trinidad donkey strain of VEEV 7 days previously (mosq) or fed upon by an uninfected mosquito (cont). Standard errors for the values averaged 0.3°C.

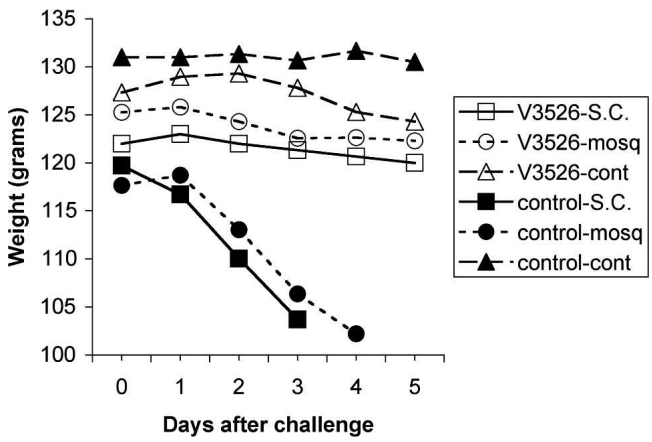

FIGURE 2. Weight (in grams) of hamsters by day after challenge with virulent Venezuelan equine encephalitis virus (VEEV). Hamsters were vaccinated with either live, attenuated V3526 vaccine (V3526) or diluent (control) 35 days before being challenged subcutaneously with $10^{3.5}$ PFU of the Trinidad donkey strain of VEEV (S.C.) fed upon by a mosquito that had been inoculated with the Trinidad donkey strain of VEEV 7 days previously (mosq) or fed upon by an uninfected mosquito (cont). Standard errors for the values averaged 1.3 g.

creased mortality[10] associated with virus introduced along with mosquito saliva than with virus inoculated alone. The mechanism for this enhancement of viral replication may be caused by pharmacologic effects of mosquito saliva. These effects include downregulation of interferon production in the presence of virus both *in vivo*[18] and *in vitro*,[19] as well as reduction of splenocyte proliferation and production of both Th1 and Th2 cytokines in the presence of mosquito salivary gland extracts.[20] However, other studies have failed to discern a difference between animals infected by needle inoculation or by mosquito bite.[21–24] This failure to detect a difference may be caused by a lack of effect of mosquito inoculation in those virus/mosquito/vertebrate combinations or an inappropriate dose for the needle inoculation. Likewise, some of the observed, enhanced effects associated with infection by mosquito bite compared with needle inoculation may be caused by using too low a dose for the needle inoculation. In our study, although all non-vaccinated hamsters challenged by either needle inoculation or mosquito bite died or became moribund between 4 and 5 days after infection, both temperatures and viremias were slightly higher in those hamsters infected by subcutaneous needle inoculation than those infected by mosquito bite. Thus, we did not find any evidence of enhancement caused by virus introduced by mosquito bite than by needle inoculation.

Because of the potential for mosquito-introduced virus to be more pathogenic, we conducted these studies to determine if the vaccine candidate would protect hamsters from the potentially more strenuous challenge of the bite of an infectious mosquito. Although both groups of mosquitoes contained essentially identical amounts of virus and were randomly allocated to the two groups of hamsters, none of the sham-vaccinated hamsters survived challenge, and all hamsters vaccinated with V3526 were protected. In addition, the second experiment demonstrated that vaccination with the V3526 vaccine candidate not only protected the hamsters from challenge with virulent virus, but also prevented the production of a detectable viremia because we did not detect virus from any of these hamsters after either needle or mosquito challenge. Similarly, there was no sign of illness in the vaccinated hamster by either route of challenge because temperatures and weights were similar to those in unchallenged controls. This finding indicates that the V3526 vaccine candidate should protect against a natural challenge with VEEV introduced by an infectious mosquito.

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